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## Multi-photon micro-spectroscopy of biological specimens

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### ABSTRACT

The non-linear nature of multi-photon fluorescence excitation restricts the fluorescing volume to the vicinity of the focal point. As a result, the technology has the capacity for micro-spectroscopy of biological specimen at high spatial resolution. Mesophyll protoplasts of *Arabidopsis thaliana* and maize stem sections were used to demonstrate the feasibility of multi-photon fluorescence micro-spectroscopy at subcellular compartments. Time-lapse spectral recording provides a means for studying the response of cell organelles to high intensity illumination.

**Keywords:** Micro-spectroscopy, multi-photon fluorescence spectroscopy, second harmonic generation, plant tissues, stem, chloroplast, protoplast, maize, *Arabidopsis*

### 1. INTRODUCTION

Due to its non-linear nature, two-photon excitation may generate very different spectral response when compared with single photon excitation. It is thus necessary to measure the two-photon spectra of samples under study, so that the two-photon fluorescence microscopic images may be properly interpreted<sup>1,2</sup>. However, fluorescence spectra obtained from bulk specimen may not provide appropriate information for microscopy. For instance, when the spectrum of a generally fluorescing specimen as a whole is obtained, a small number of fluorescent particles may escape from detection due to the relatively small contribution to the total fluorescence. In addition, signals resulted from second harmonic generation (SHG) may be mixed with low level broad band background autofluorescence which is commonly found in biological specimen. Therefore, measuring fluorescence spectrum from a micro-focused volume is essential to properly interpret multi-photon fluorescence microscopic images. Under two-photon excitation, the background noise may be greatly reduced due to the naturally limited excitation volume of the focused laser beam. In this study, leaf protoplasts of *Arabidopsis thaliana* and stem slices of maize (*Zea mays*) were used as samples to address these issues in a set-up for micro-spectroscopy.

### 2. MATERIALS AND METHODS

Protoplasts of *Arabidopsis thaliana* were isolated from leaves through enzyme digestion of the cell wall, and suspended in culture medium according to the methods of Huang and Chen<sup>3</sup>. For microscopic observation, the protoplast suspension was placed in a chambered coverglass (Lab-Tek, Illinois, USA). Unstained and Calcofluor White-stained stem slices of maize were also used.

Two-photon induced fluorescence spectra were measured with two sets of spectrometers. For static spectra, a SpectraPro-500 spectrometer (Acton Research) equipped with a TE-cooled PMT is used to achieve higher spectral precision. Whereas for time-lapse spectra, a 1/8m spectrometer (Oriel, #77250) equipped with a liquid-nitrogen-cooled CCD camera is used for high speed spectrum acquisition. The excitation laser beam is derived from a Spectra Physics Tsunami Ti:sapphire laser pumped

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by a Coherent Verdi solid-state laser at 532nm. The Ti:sapphire laser is mode-locked at 780nm and has a repetition rate of 82 MHz with a pulse width of approximately 100fs. A ChromaTech dichroic beam splitter (650DCSP) was used to achieve epi-illumination and on-axis fluorescence detection in a modified Olympus BX microscope. In addition, two IR cut-off filters (Edmond Scientific, Cat. K53-710) were installed in front of the entrance slit of the monochromator to reject scattered IR from the sample. Using this set-up, site-specific spectral information was obtained from the samples<sup>4</sup>. Excitation intensity as high as  $10^{12}$  W/cm<sup>2</sup> was reached at the focal point. A second set-up using a Spectra-Physics Millennia IR (1064nm) pumped Chromium-doped Forsterite laser (built by CKS), operated at 120MHz and 130fs pulse, was used for 1234nm infrared (IR) excitation. Figure 1 is a diagrammatic representation of our micro-spectroscopy set-up.

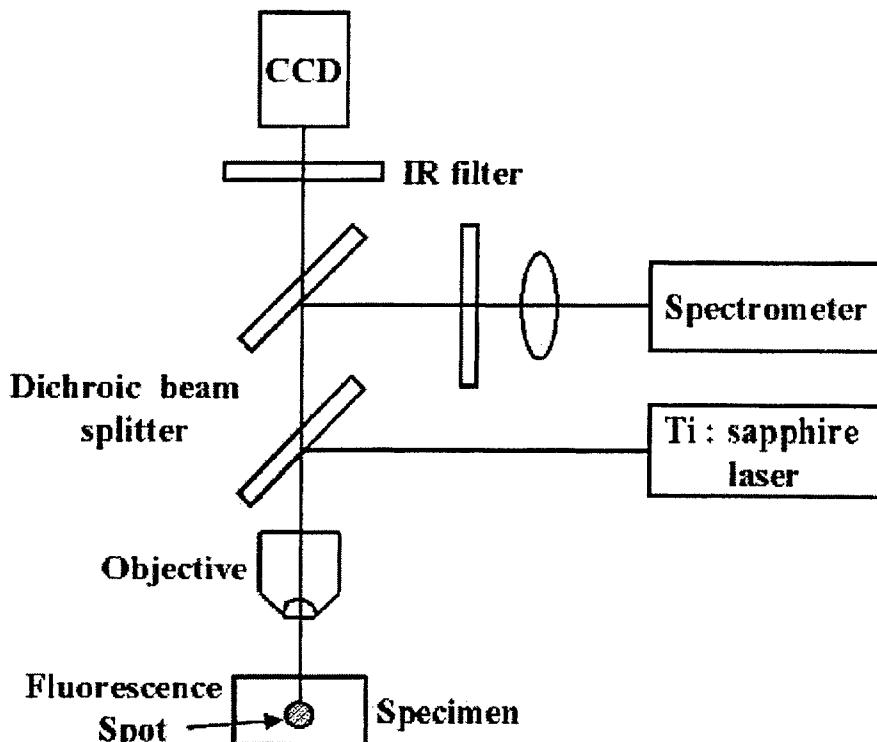


Figure 1. Diagrammatic representation of micro-spectroscopy set-up.

### 3. RESULTS AND DISCUSSION

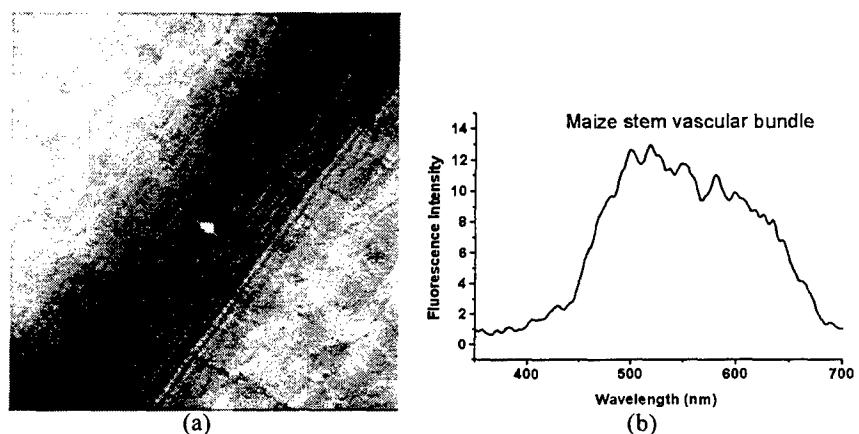
Figure 2a is a transmission image of a maize stem showing cortical parenchyma cells and a vascular bundle. An IR beam was tightly focused on a region of a cell in the vascular bundle and generated a fluorescence spot. Two-photon excited spectrum taken from this spot shows a broad band fluorescence peaked approximately at 500nm (Figure 2b). When stained with Calcofluor White, a fluorescent dye staining cellulose, the cortical parenchyma cell wall shows two-photon fluorescence (Figure 3a), while the emission spectrum has the characteristics of Calcofluor White (Figure 3b). These results demonstrate the potential of micro-spectroscopy in studying fluorescence emission properties of subcellular compartments. Because of the non-linear properties of the two-photon fluorescence excitation, this technique provides superb spatial resolution in 3D.

Figures 4a and 4b are two-photon fluorescence micrographs of *Arabidopsis thaliana* mesophyll protoplasts. In these micrographs, the center region received a single 3.3sec/frame scan (8.4μs dwell time/pixel) at 768x512 pixels. The total irradiated area is 180μm x 120μm. At focal point, the average power measured was 6.4 mW, therefore, the average energy exerted on the specimen for each pixel is approximately 54nJ (obtained by 8.4μs X 6.4mW). On the other hand, considering the laser operating at 82 MHz with 100fs pulse, when a NA=1.2 objective lens was used, the average and peak power densities at the focal point approximate  $3 \times 10^6$  W/cm<sup>2</sup> and  $3.9 \times 10^{11}$  W/cm<sup>2</sup>, respectively. After the first scan, it was noted that a significant reduction in red fluorescence occurred, as evident when using different filters (comparing Figure 4a with 4b). The images were taken at a lower magnification to show the scanned area. Figures 4c and 4d show fluorescence images

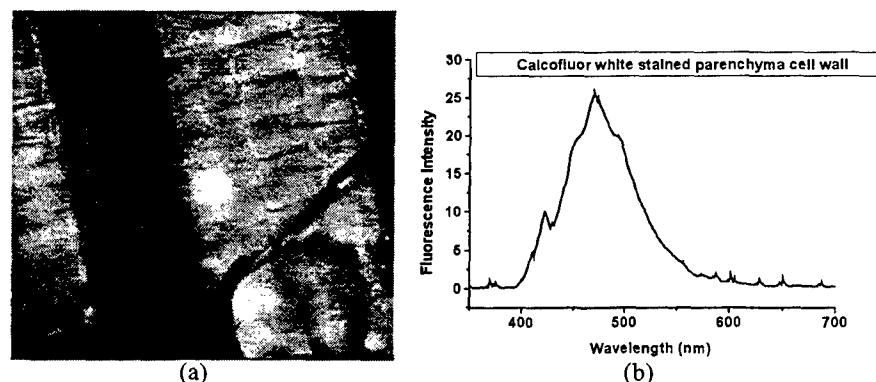
obtained from the 1<sup>st</sup> and 3<sup>rd</sup> scan. Though the fluorescence intensity is seen to reduce after several scans, the images do not provide detailed information on the possible spectral variation.

Figure 5b shows a three-dimensional plot of spectra against time indicating the spectral and intensity changes of the green autofluorescence from a chloroplast under intense NIR illumination (Figure 5a). In addition to the broad band green fluorescence emission, peaked at 532nm/550nm (Figure 5b), a prominent red fluorescence peak with emission maximum at 663nm has been observed in a previous study<sup>2</sup>. The 663nm emission is the result of chlorophyll fluorescence while the origin of the 532/550nm emission is yet to be determined<sup>2</sup>. These results demonstrate that the set-up for micro-spectroscopy allows time-lapse recording in subcellular organelles, and thus will be useful for detailed spectral analysis of possible cellular damages or photochemical reactions under multi-photon excitation.

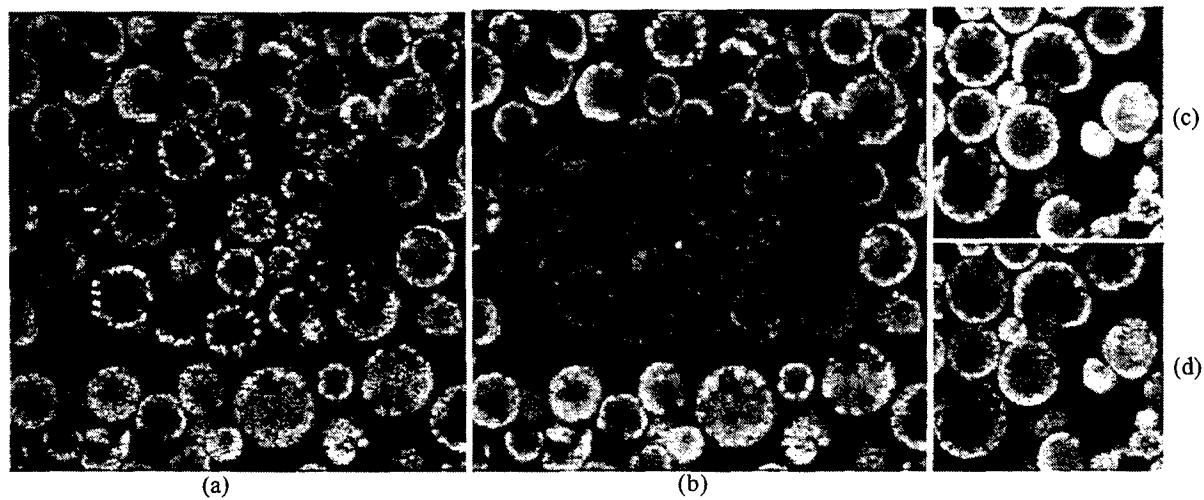
Micro-spectroscopy also provides insights into the origin of signals detected in a multi-photon microscopic imaging system. For instance, Figure 6 shows an emission spectrum obtained from the cortex of a maize stem. When excited with 1234nm IR, a red fluorescence peak at 682nm and a small peak at 617nm were observed. The red peak originates from the auto-fluorescence of chlorophyll. The smaller peak is likely the result of second harmonic generation (SHG) in the plant cell wall. In this case, micro-spectroscopy provides a means of verification that, indeed, SHG occurs in plant samples<sup>5</sup>.



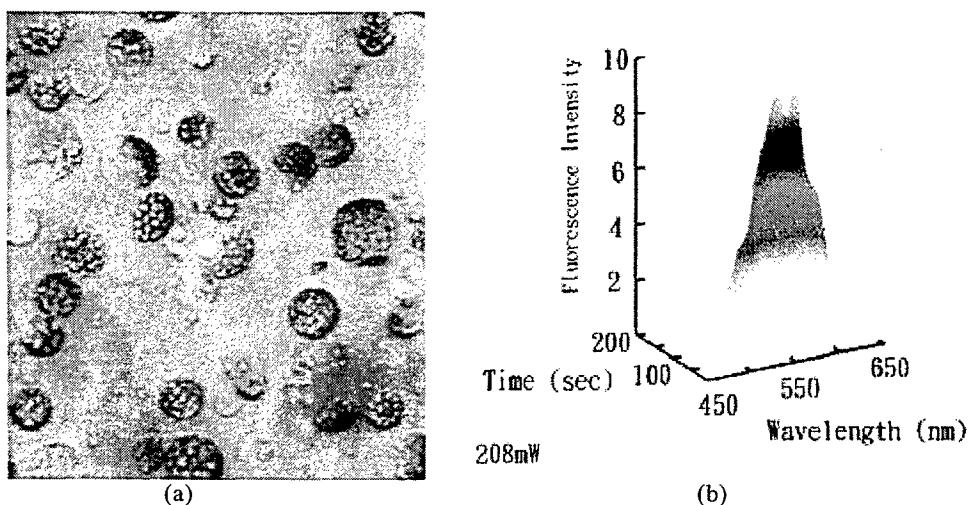
**Figure 2** (a) Transmission micrograph of a longitudinal section of maize stem. The bright spot in the center of the micrograph indicates two-photon excited fluorescence emission generated by a tightly focused IR beam. (b) The fluorescence spectrum obtained from the spot shown in (a).



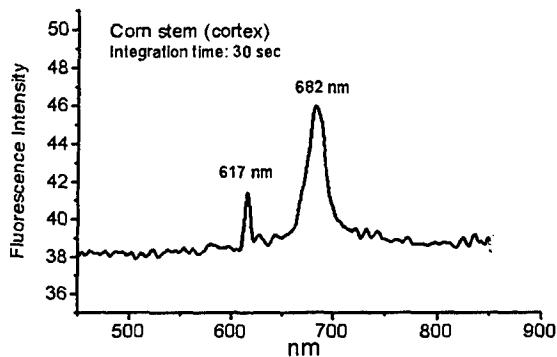
**Figure 3.** (a) Transmission micrograph of a longitudinal section of Calcofluor White-stained maize stem. The large bright spot in the center of the micrograph is two-photon excited fluorescence emission generated by intense IR beam. (b) The fluorescence spectrum obtained from the spot shown in (a).



**Figure 4.** (a) and (b) two-photon fluorescence images showing a previously scanned area in the center. In (a), obtained with a green filter, the scanned area is brighter, whereas in (b), obtained with a red filter, the scanned area is dimmer. The contrast in fluorescence intensity is induced by a single imaging scan. (c) and (d) sequential images obtained from the 1<sup>st</sup> and 3<sup>rd</sup> scan, respectively. In (c) and (d), both green and red fluorescence images are superimposed.



**Figure 5.** (a) Transmission micrograph of *Arabidopsis thaliana* protoplasts. The bright spot in the center is the fluorescence emission generated by a tightly focused NIR beam. (b) Three-dimensional plot of fluorescence emission spectra vs. time showing changes in spectrum and intensity of fluorescence from within a protoplast of *Arabidopsis thaliana* as shown in (a).



**Figure 6.** Spectrum obtained from the cortex in maize stem cross-section excited by 1234nm IR. Note the red fluorescence at 682nm and SHG at 617nm.

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